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Lead article

Identification of a 1.2 Kb cDNA fragment from a region on 9p21 commonly deleted in multiple tumor types

Susan Gursky,[a,1] Olufunmilayo I. Olopade,[b] Janet D. Rowley[b,*]

[a]*Department of Biochemistry and Molecular Biology, 5841 South Maryland Avenue, MC 2115, The University of Chicago, Chicago, IL 60637, USA*
[b]*Section of Hematology/Oncology, Department of Medicine, 5841 South Maryland Avenue, MC 2115, The University of Chicago, Chicago, IL 60637, USA*

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Abstract Chromosome band 9p21 is a frequent target of homozygous deletion in many tumor types. Putative tumor suppressor genes, *CDKN2A* (p16), $p14^{ARF}$ and *CDKN2B* (p15), were localized to 9p21. However, there have been reports that suggest that there may be other genes targeted for inactivation in the region. We have developed a method to search for transcribed sequences within large genomic regions. We tested our approach in a 100-kilobase region on 9p21, which is 40 kilobases telomeric to *CDKN2A*. The method, termed expressed sequence selection (ESS), resulted in the isolation of genomic fragments known to be from 9q21 that are homologous to transcribed sequences. One fragment was used to obtain a 1.2 kilobase cDNA. The sequence of the 5′ half of the cDNA was almost identical to exons 3–5 of the *MTAP* gene, which maps to chromosome band 9p21. The 3′ portion of the cDNA had sequence homology to the *ALA* gene, which maps to chromosome arm 9q. Using Northern blot analysis, the 1.2 Kb cDNA identified several widely expressed transcripts ranging from 1 Kb to 8.5 Kb and displayed a complex pattern of alternative splicing in which certain exons of the 1.2 Kb cDNA are excluded from some of the splice products. Using cancer tissue Northern blots, we could show that all of the transcripts are absent from a leukemia cell line and a lung cancer cell line (K562, A549) with homozygous, genomic deletions within chromosome band 9p21. In addition, the 7 Kb transcript is also absent from two additional tumor cell lines (Molt4, a leukemia derived cell line, and in G361, a melanoma derived cell line) with homozygous deletions. Further investigation will determine whether the difference in the expression pattern between the 7 Kb transcript compared with the other sized transcripts could be due to specific targeting for alteration in certain tumor types.

1. Introduction

Submicroscopic homozygous deletions of chromosome band 9p21 in human malignancies were first identified by the loss of the interferon gene cluster in primary leukemia cells and derived cell lines [1]. Researchers subsequently found homozygous deletions of 9p21 in both solid tumors and leukemia [1–5]. The search for tumor suppressor genes in this region has resulted in the isolation of putative tumor suppressor genes (TSGs), *CDKN2A/p16* (and subsequently, an alternative splice product $p14^{ARF}$) and *CDKN2B/p15* [6]. These genes are deleted in tumors with structural abnormalities of chromosome band 9p21. However, there is evidence suggesting that there may be other TSGs in the region. One observation was the high incidence of loss of heterozygosity (LOH) without alterations in either *CDKN2A* or *CDKN2B* [7–10]. Another observation was a much higher incidence of homozygous deletion of these genes in cell lines as compared with primary tumors [10–13]. Lastly, there have been reports of regions of deletion on 9p21 that exclude *CDKN2A/B* in several tumor types [14–18]. These data have led to the proposal that other TSGs map to the region.

To isolate other genes in the 9p21 region, we used a technique we termed express sequence selection (ESS) (Fig. 1) to analyze a 100 Kb cosmid contig that mapped approximately 40 Kb telomeric to *CDKN2A* (Fig. 2). We have observed that the 9p21 band is repeat-dense and this can cause difficulty isolating region specific genes. Therefore, we have developed the ESS technique to solve the problems as-

* Corresponding author. Tel.: 773-702-6117; fax: 773-702-3002.
E-mail address: jrowley@medicine.bsd.uchicago.edu (J.D. Rowley).
[1]Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014.

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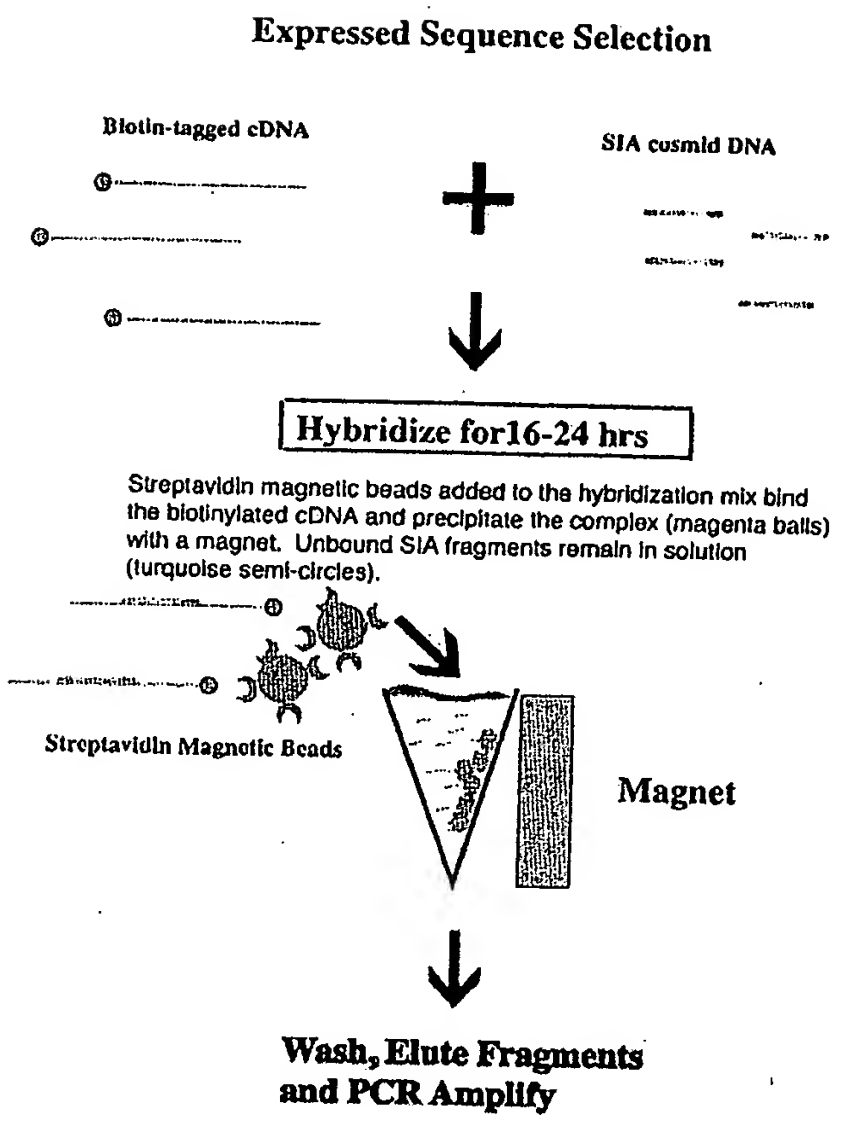

Fig. 1. The expressed sequence selection technique. Step 1: Biotinylated cDNA and genomic DNA that have been suppressed for repetitive sequences are hybridized. Step 2: The biotinylated cDNA and the bound genomic fragments are captured by the magnetic beads. Step 3: The complexes are washed stringently, and the remaining genomic fragments are eluted. Step 4: The eluted genomic fragments are amplified by PCR.

sociated with isolating genes in repeat-dense regions. The technique is described in detail [19]. Genomic fragments from 9p21 that identified transcripts by Northern blot analysis were isolated. One of these fragments was chosen for further characterization based on its ability to identify widely expressed, 9p21-specific transcripts. Here we describe the partial characterization of a 1.2 Kb cDNA identified by the expressed genomic fragment (Genbank accession number AF216650).

2. Methods

2.1. Experimental design

ESS is based on the direct sequence selection technique [20]. However, difficulties associated with this and other hybridization-based techniques are most prevalent when isolating genes from repeat-dense regions. Frequently, cDNA is selected based on sequence homologies in repetitive region, and generally do not map to the region of interest. Therefore, to isolate region-specific cDNA, we first hybridize cloned genomic DNA to cDNA inserts. The region-specific genomic fragments bind the cDNA inserts and are amplified by PCR. The sequences of these fragments are used to design primers to amplify region-specific cDNA (Fig. 1).

2.2. Preparation of cDNA inserts

Ten microliters (10^7 pfu) each of a human adult brain and a human foreskin fibroblast cDNA library in lambda gt11 (Clonetech, directly from phage stock) were boiled for 5 min and each library was specifically biotinylated at the 5′ end of each molecule through PCR. Vector primers with a 5′ biotinylated nucleotide (**B**) on the reverse 3′) primer used were: 5′-GGTGGCGACGACTCCTGGAGC-3′; 5′-**B**-GACACCAGACCAACTGGTAATG-3′. The PCR reaction was carried out in a 100 μl reaction consisting of 50 mM KCl, 10 mM Tris-HCL, pH 8.3, 1.2 mM $MgCl_2$, 0.01% gelatin, 125 mM each deoxynucleotide, 1 mM of each vector primer listed above, and 2.5 U Taq polymerase (Perkin-Elmer). The cDNAs were denatured for 2 min at 94°C, and amplified using 40 cycles (1:00, 94°C; 1:00, 62°C; 4:00, 72°C), followed by a 7-min extension at 72°C in a 9600 Thermocycler (Perkin-Elmer-Cetus). The amplification products were precipitated with 2 M ammonium acetate and 2.5 volumes of 100% ethanol, which does not effectively precipitate unincorporated nucleotides and primers. One-half of each PCR reaction (≈2.5 μg) and 50 μg Cot 1 DNA were combined, denatured, and prehybridized in 50% formamide solution (50% formamide, 20% dextran sulfate, 1M NaCl, 1% sodium dodecyl sulfate, 0.5 M sodium phosphate pH 6.5) for 30 min at 37°C in a total volume of 100 μl. This solution was used for the hybridization with the region specific cosmids that have undergone sequence-independent amplification (SIA). The region specific cosmids were derived from YAC 807E4 (CEPH MegaYAC library).

2.3. Preparation of SIA-cosmids

Genomic DNA from a cosmid pool from the specific region of interest on 9p was used as the template for SIA as previously described [21]. The cosmids were mapped to 9p21 by fluorescence in situ hybridization [22]. The cosmids were obtained from a chromosome 9 cosmid library (a gift from Oncor Med, Inc. and Lawrence Livermore Laboratory). SIA products (400 μg) were preannealed with a cocktail to suppress hybridization of vector sequences and repetitive sequences. The cocktail consisted of Lawrist 16 vector (3 μg) digested with Ban I restriction endonuclease, phenol-chloroform extracted once and ethanol precipitated, Cot 1 DNA (50 μg), and sheared placental DNA (50 μg). The cocktail and SIA-cosmids were boiled for 2 min then annealed in 100 μl of the formamide solution (described previously) for 30 min at 37°C. This mixture was added to the prehybridized cDNA inserts (as described) and hybridized at 42°C for 24 h (200 μl total volume).

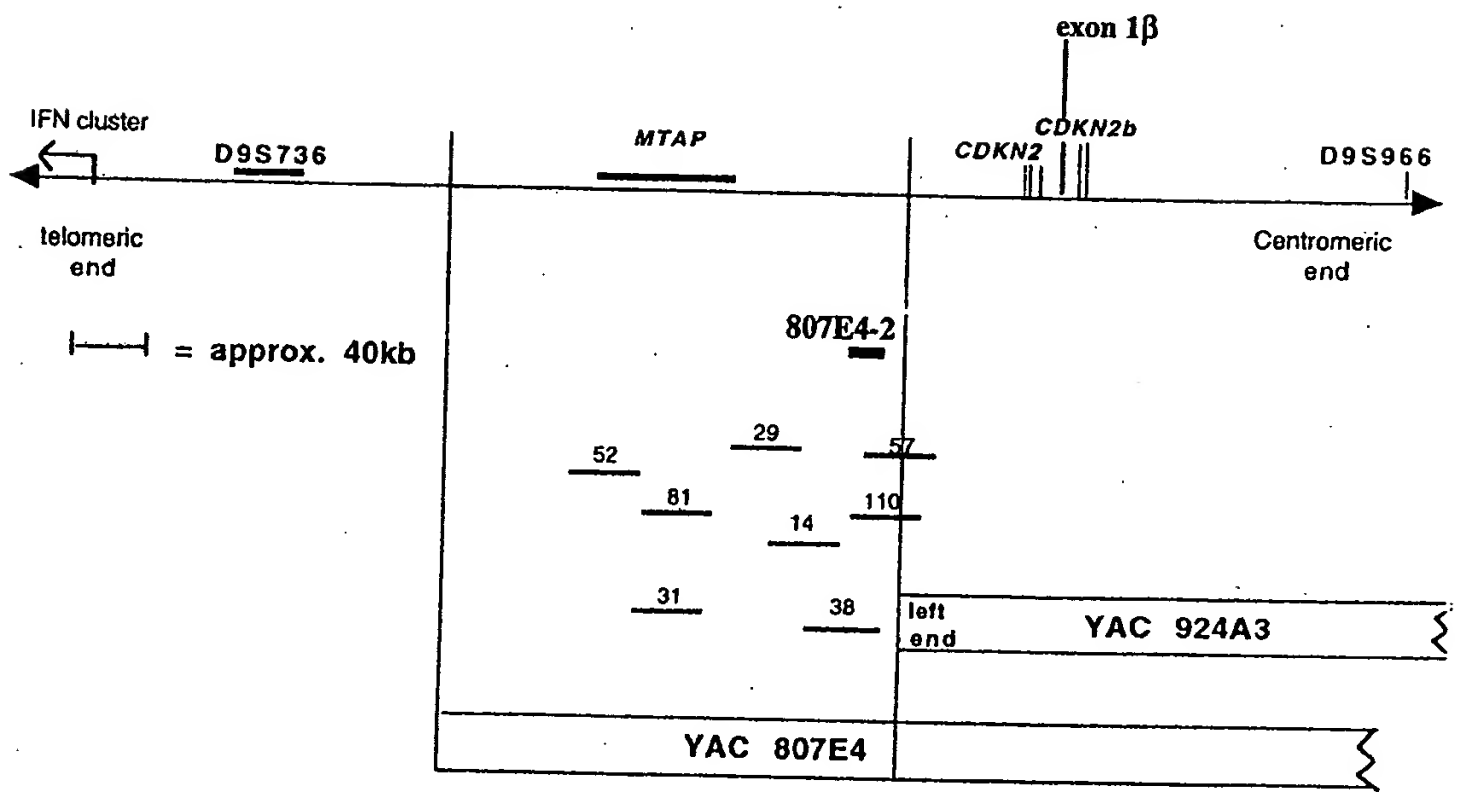

Fig. 2. Map of the genomic, cosmid and YAC DNA on chromosome band 9p21. The genomic map of regional genes and sequence-tagged stie:: The cosmid contig used in the ESS procedure is represented by thin lines with individual cosmid numbers above. Expressed sequence 807E4-2 is represente.! by the thick line, and YACs are boxed.

2.4. Preparation of streptavidin magnetic beads and binding to the biotinylated cDNA/cosmid DNA hybridization mixture

Two hundred milliliters of streptavidin magnetic beads (Dynal) were prepared according to the manufacturer's instructions. The magnetic beads in solution were added to the hybridization mixture (as described), and gently agitated for 1 h at room temperature. The high salt concentration of the solution allows the binding of the streptavidin conjugated magnetic beads to the biotinylated cDNA. The complexes containing the streptavidin magnetic beads, biotinylated cDNA, and the genomic fragments that bind the cDNA were collected by magnetic separation and the supernatant was removed. The complexes were then washed: 15 min, room temperature in 2×SSC; 15 min at 68°C in 2×SSC; two washes, 30 min each, 68°C in 0.5×SSC. After each wash, the complexes were separated from the solution with a magnet and the solution was discarded. The complexes then were resuspended in 21μl sterile ddH_2O and heated to 72°C for 10 min to elute genomic fragments that were bound to the cDNA.

2.5. Amplification of the selected genomic fragments

Eluted genomic fragments (above) were amplified by PCR. PCR cycles were as follows: The step cycle consisted of six initial rounds of PCR of 94°C, 30 s; 56°C, 30 s; 72°C, 2 min; followed by 35 rounds with an annealing temperature of 62°C, 30 s (denaturation and elongation as described). Concentrations of the reaction mix were the same as above, except 2.2 mM of primer B-CUA (primer B-CUA contains 5′ sequences, which allows efficient cloning using the pAMP1 cloning system [Life Technolog}, 5′CUA CUACUACUAAGAGTTGGT AGCTCTTGAT C3′) was used in a 30 μl reaction. PCR products wre electi ophoresed on a 1% agarose gel, along with a negative contr l PCR reaction (which ensures that the random-primed SIA fragments that were amplified were derived from th selection procedure and not a laboratory contamination). PCR products were cloned into pAMP1 and used to transfor n DH10β competent cells (Stratagene) using standard procedures [16]; the bacteria were selected on LB/ampicillin plates. Individual colonies were isolated and amplified by PCR using primer B (5′AGAGTTGGTAGCTCTTGATC3′) for 30 cycles of 30 s at 94°C; 30 s at 56°C; and 40 s at 72°C, with reagent concentrations listed previously. The products were electrophoresed on a 1.2% agarose gel.

2.6. Analysis

PCR products from the individual clones were labeled by random oligonucleotide priming with 5 μCi of α-^{32}P [23] and were used as probes on multiple tissue Northern blots, cancer tissue Northern blots (Clonetech) and a bladder cancer Northern blot using standard procedures [24]. Bladder cancer mRNA was a kind gift from Dr. Walter Stadler, University of Chicago. The loading of DNA or RNA was shown to be approximately equal for all blots as seen by the hybridization of a control β-actin probe (data not shown). Primers from selected fragment 807E4-2 (pr1 5′GGC AGTCTGTCCACATGTGC3′; pr2 5′AAGTCCT GAAGC TGGTGATCA3′) were used to amplify cDNA from a placenta RACE-ready cDNA library (Clonetech), according to manufacturer's instructions. PCR reagents were as described previously and reaction conditions were as follows:

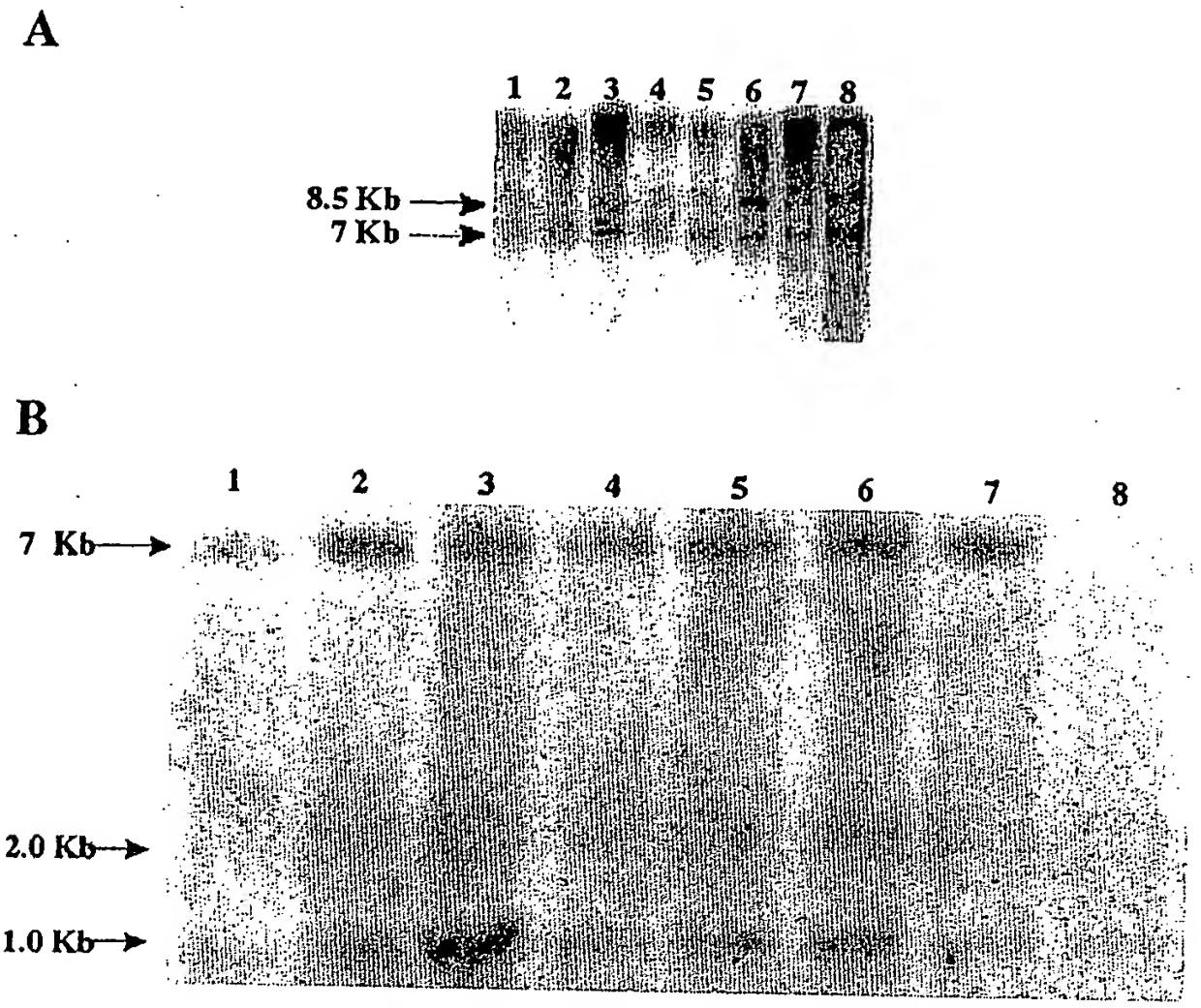

Fig. 3. Northern blot hybridization of expressed-sequence 807E4-2. The probe was labeled with 5 μC of ^{32}P. (A) Tissue specificity of 807E4-2 on a multiple tissue Northern blot. Lane 1, heart; Lane 2, brain; Lane 3, placenta; Lane 4, lung; Lane 5, liver; Lane 6, skeletal muscle; Lane 7, kidney; Lane 8, pancreas. (B) Expression pattern in bladder tumor cell lines. 253J (lane 8) is the only cell line with a homozygous deletion of 9p21, and the deletion breakpoint maps within the cosmid contig. Lane 1, SCAber; Lane 2, HT1396; Lane 3, HT1197; Lane 4, 5637; Lane 5, T24; Lane 6, 647V; Lane 7, 639V; Lane 8, 253J.

94°C, 30 s followed by 25 rounds of 94°C, 20 s; 68°C, 10 s; 62°C, 5 s; 68°C, 10 min. Products were electrophoresed on a 1% agarose gel, and then stained with ethidium bromide. One RACE product (1.2 Kb) was isolated from the gel and purified with a spin column (Qiagen) according to manufacturer's instructions. The isolated cDNA was used as a template for SIA. The resulting SIA fragments from the 1.2 Kb cDNA were cloned and sequenced in the same manner as the SIA-cosmid fragments from the selection procedure.

3. Results

3.1. Analysis of the expressed fragments

All six clones isolated during the ESS procedure were found to map to 9p21 cosmids and identified transcripts on multiple tissue Northern blot analysis (data not shown). One fragment, 807E4-2 (Fig. 2), was of particular interest because it identified large transcripts that were widely expressed (Fig. 3A). A 7 Kb transcript identified by 807E4-2 was absent from a bladder tumor cell line (253J) with a relatively small deletion in the CDKN2 region (Fig. 3B, lane 8), but was present in cell lines with no known deletions in the region (Fig. 3B, lanes 1–7). This expression pattern suggested that the transcript is located on 9p21.

3.2. Northern blot analysis of a 9p21-derived cDNA

To obtain a cDNA of the gene identified by 807E4-2, primers from this sequence were used to amplify cDNAs from a RACE-ready placenta cDNA library (Clonetech). Two cDNAs (3.5 Kb and 1.2 Kb) were isolated from the RACE reaction. The 3.5 Kb cDNA identified a widely expressed 9Kb transcript that was absent from 7/16 (66%) tumor cell lines whereas the transcript was present in normal tissue from which the tumors were derived. However, the cDNA did not map to the cosmid contig (data not shown). We assumed the 3.5 Kb cDNA was part of a gene homologous to the gene contained in 807E4-2, but which mapped elsewhere in the genome. We then isolated and analyzed a cDNA that was approximately 1.2 Kb and determined that it was located on 9p because of strong hybridization to cosmid DNA from the region (data not shown). The cDNA mapped to a group of cosmids (c38, c110 and c57) located near the left end of YAC 924A3, the same cosmids to which the 807E4-2 probe mapped (see the map of the cosmids in Fig. 2). In addition, the cDNA mapped to c52, c31 and c81, located at the telomeric end of the cosmid contig, but did not hybridize to c29 and c14 located in the middle of the contig. This suggested that the 1.2 Kb cDNA was a mature transcript that had been spliced, and that the cDNA maps to the cosmid contig on 9p.

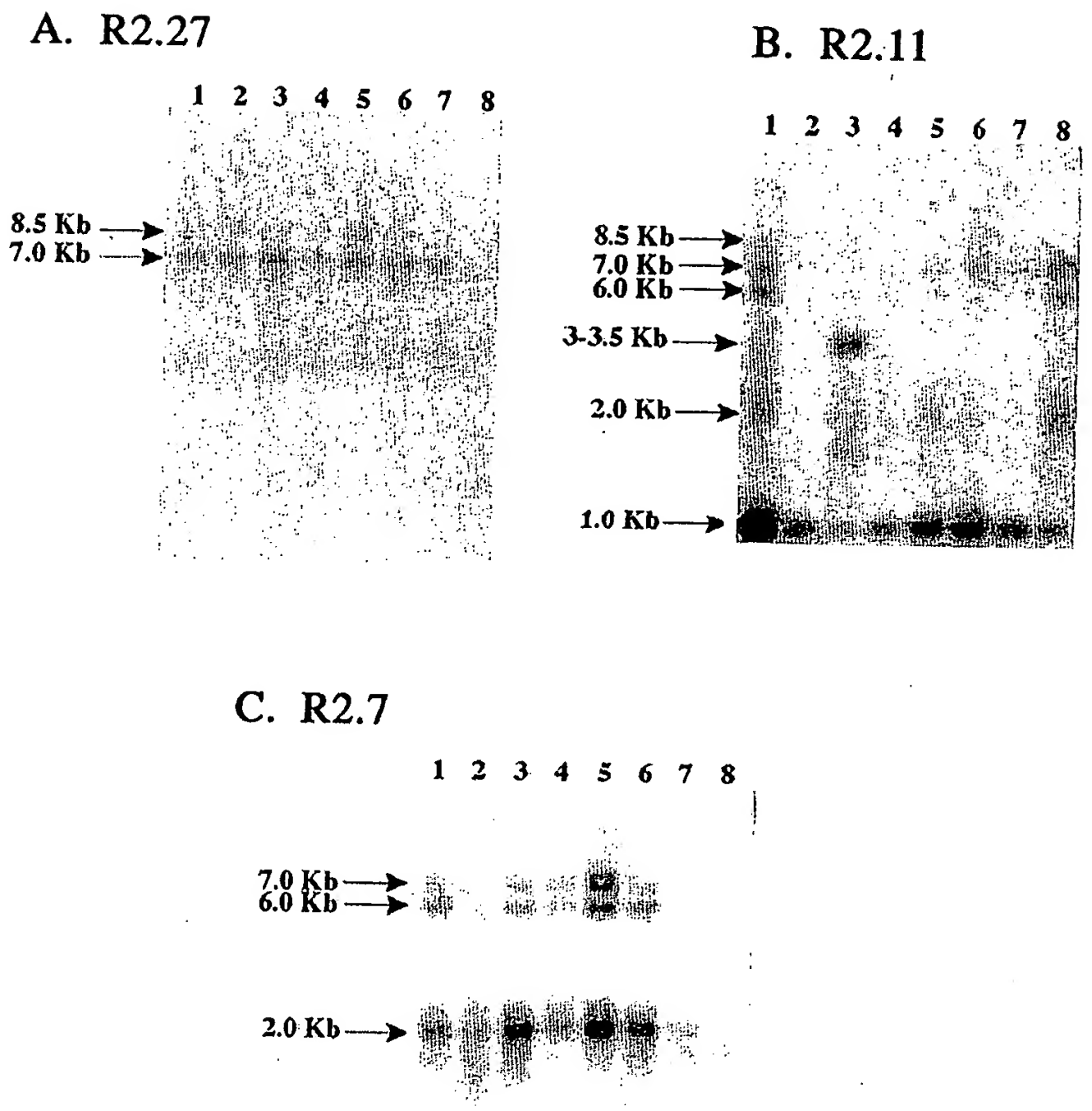

Fig. 4. Northern blot hybridization of three subclones derived from the 1.2 Kb cDNA. Tissue specificity of the 1.2 Kb cDNA and the transcript sizes of the three subclones of the cDNA. Subclone R2.27 and R2.7 correspond to distinct exons in the cDNA. The R2.11 sequence overlaps the sequences of R2.27 and R2.7. The probes were labeled with 5 μC of ^{32}P. (A) Subclone R2.27 hybridization. Lane 1, heart; Lane 2, brain; Lane 3, placenta; Lane 4, lung; Lane 5, liver; Lane 6, skeletal muscle; Lane 7, kidney; Lane 8, pancreas. (B) Subclone R2.27 hybridization. Lane 1, spleen; Lane 2, thymus; Lane 3, prostate; Lane 4, testes; Lane 5, ovaries; Lane 6, small intestines; Lane 7, colon; Lane 8, PBL. (C) Subclone R2.11 hybridization. Lane 1, heart; Lane 2, brain; Lane 3, placenta; Lane 4, lung; Lane 5, liver; Lane 6, skeletal muscle; Lane 7, kidney; Lane 8, pancreas. (D) Subclone R2.7 hybridization. Lane 1, heart; Lane 2, brain; Lane 3, placenta; Lane 4, lung; Lane 5, liver; Lane 6, skeletal muscle; Lane 7, kidney; Lane 8, pancreas.

Three subclones of the 1.2 Kb cDNA (R2.27, R2.11, R2.7) were hybridized to multiple tissue Northern blots to determine whether the cDNA detects the 7 Kb and 8.5 Kb transcripts detected with the 807E4-2 probe (Fig. 4A–C). R2.27 displayed the same pattern as 807E4-2, binding to both the 7 Kb and 8.5 Kb transcripts, but also to a 3–3.5 Kb transcript in placenta and a 2 Kb transcript in pancreas (Fig. 4A). R2.11 hybridized to the 7Kb and 8.5 Kb transcripts, a 3–3.5 Kb transcript in placenta plus two widely expressed transcripts (6 Kb and 2Kb), and a strongly expressed 1 Kb transcript (Fig. 4B). R2.7 detected the 7 Kb transcript (but not the 8.5 Kb transcript), the 6 Kb and 2 Kb transcripts seen with R2.11, plus an additional 2.8 Kb transcript (Fig. 4C). A 7.5 Kb transcript was also detected in ovarian tissue (Fig. 4C, lane 5). Similar to the transcripts identified with 807E4-2, most of the extra transcripts identified were widely expressed. All three subclones hybridize to the 7 Kb transcript, but the 5′ region of the cDNA (R2.7) identifies transcripts that do not hybridize to the 3′ region (R2.27). These differences might be due to the hybridization of the alternative splice products of the gene.

R2.7 was hybridized to a cancer tissue Northern blot. All of the transcripts that were identified by R2.7 in the multiple tissue Northern were absent in K562 (a cell line with a very large homozygous deletion of 9p21) and A459 (a cell line with a small homozygous deletion of 9p21) (Fig. 5, lanes

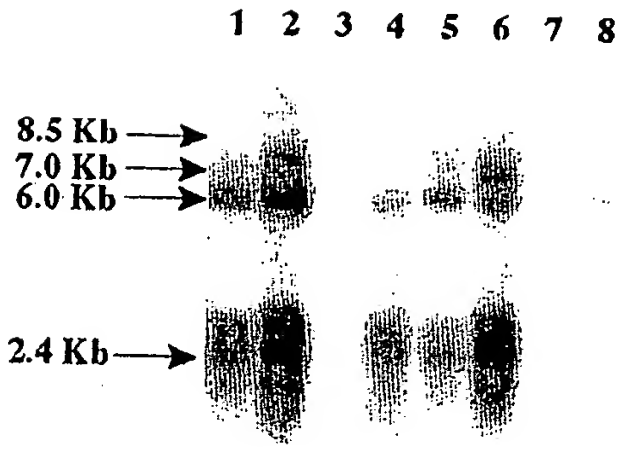

Fig. 5. Cancer tissue Northern blot hybridization of the R2.7 subclone derived from the 1.2 Kb cDNA. Cancer tissue specificity of the 1.2 Kb cDNA and the transcripts sizes of the R2.7. The probe was labeled with 5 μC of ^{32}P. Lane 1, HL60; Lane 2, HeLa; Lane 3, K562; Lane 4, Molt4; Lane 5, Raji; Lane 6, SW40; Lane 7, A549; Lane 8, G361.

3,7). This indicates that all the transcripts map to 9p21. The 7 Kb transcript detected with R2.7, R2.11, R2.27, and 807E4-2 was not seen in MOLT 4 or G361 (both cell lines have small deletions on 9p21), but other 9p-specific transcripts are present in those two cell lines (Fig. 5, lanes 4,8). This indicates that the 7 Kb transcript is altered more frequently in these cell lines than the other transcripts and may be a more sensitive indication of small deletions in this region.

3.3. Sequence analysis of the 1.2 Kb cDNA

Fig. 6 shows the sequence of the 1.2 Kb cDNA. The 5′ end of the sequence (italics) shows sequence identity to the *MTAP* (methylthioadenyl phosphorylase) gene, a gene contained in a 2.5 Kb cDNA. The sequence diverges from the exon 5/6 junction of *MTAP*, which is located in c81. The 3′ end of the sequence showed sequence identity with c110 (underlined) and sequence homology to an unpublished gene in Genbank (*ALA*), but no identity in this region with *MTAP*. This results in the exchange of the terminal 67 amino acids of *MTAP* for 63 new amino acids. Cosmid 110 contains a putative AG splice acceptor site adjacent to the region of homology to the cDNA (data not shown). The largest open reading frame of the cDNA ends at the asterisk and the translation of which appears below. The 1.2 Kb cDNA did not appear to contain the transcriptional or translational start site.

We made numerous attempts to obtain a full-length cDNA both by RACE reactions and by screening several cDNA libraries. Although we amplified larger cDNA (the largest one was between 4 and 5 Kb), we were unable to subclone them. this was due in part to the fact that cDNA amplification is less efficient with long molecules than smaller ones. We believe that the ends of the cDNA were damaged in the purification process, which made reamplification impossible. Also primer design is important for efficient amplification and our sequence shares either sequence identity or sequence homology to other genes. There is no continuous portion of the sequence completely unique, and therefore designing specific primers that produced robust amplification is difficult. Hybridization to a cDNA library was also inefficient because the *MTAP* region hybridized to the highly expressed 2.4 Kb *MTAP* transcript. The 3′ end hybridized to an unpublished gene (*ALA*) and its homologues.

4. Discussion

Structural abnormalities of chromosome band 9p21 in tumor cells are usually homozygous deletions. Homozygous deletion rather than point mutation could have a selective advantage if more than one TSG was located in the region, and inactivation of both was required of tumorigenesis. Neither TSG would be expected to be deleted more frequently than the other. However, this is not seen with *CDKN2A* and *CDKN2B*. In every tumor type tested thus far, *CDKN2A* is always deleted or mutated more frequently than *CDKN2B* or $p14^{ARF}$, even though $p14^{ARF}$ shares its second exon with CDKN2A [25]. In fact, the mutations in exon 2 found in human tumors that have been tested for p16 and p14 inhibitor function have shown a disruption in p16 exclusively [26]. This may occur because in the mouse homologue of p14 (p19), the first exon is sufficient to induce cell cycle arrest [25]. Moreover, point mutations in the human exon1β have not been identified [26]. It appears that among these three genes, only *CDKN2A* is a major target for alteration in human tumors, despite the fact that $p14^{ARF}$ seems to play a more crucial role in cell cycle control (for review, [27]). A study of 545 primary tumors identified a 170 Kb common region of deletion that included *CDKN2A* and extended telomerically, but excluded *CDKN2B* [28]. This suggests that there may be another target for deletion telomeric to the CDKN2 locus. Therefore, isolation of more transcripts from this biologically and genetically interesting region is important.

Here, we describe a widely expressed 1.2 Kb partial cDNA mapping approximately 40–50 Kb telomeric to *CDKN2B*. Because structural abnormalities of chromosome band 9p21 are present in a wide variety of tumor tissues, we would also expect a TSG in this region to have a broad tissue expression. Transcripts identified by the 1.2 Kb cDNA were present in every tissue tested except peripheral blood leukocytes and they displayed a complex pattern of tissue expression (Fig. 4A–C). The most likely explanation for the hybridization pattern is that the cDNA is a transcript from a gene that has several alternatively spliced variants. As expected, probes from the 1.2 Kb cDNA located in the region of identity with *MTAP* hybridized to the same size transcripts previously identified by the *MTAP* cDNA (cf. Fig. 4C with [29]). However, probes from the c110 region of the cDNA bind an 8.5 Kb transcript not seen with the *MTAP*-like probes (Fig. 4A and B). This suggests that this transcript has limited sequence in common with the previously published *MTAP* gene. However, we do not know whether the 1.2 Kb cDNA is a distinct gene that uses some of the

AAAAATKTTRATTGCATCCYCCTTGCAAGGCATGGAAGGCAGCACACCATCWTGCCTTCAAAG
K N X X C F X L A R H G R Q H T I X P S K

GTCAACTACCAGGCGAACWTCTGGGCTTTRAAGGAAGAGGGCTGTACACWTGTCWTAGTRAC
V N Y Q A N X W A L K E K G C T X F X L T

CACASCTTGTGGCTCCTTGAGGGAGGAGATTCACCCCGGCRCTATTGTCATTATTGATATGTTC
T W C G S L R E E I H P G X I V I I D M F

ATTGACAGGACCACTATAAGGCCTCAGTCCTTCTATGATGGAAGTCATTCTTGTGCCAGAGGAG
I D R T T I R P Q S F Y D G S H S C A R G V

TGTGCCATATTCCAATGGCTGAGTCGTTTTGCCCCAAAACGAGAGAGGTTCTTATAGAGACTGC
C H I P M A E S F C P K T R E V L I E T A

TAAGAAGCTAGGACTCCGGTGCCACTCAAAGGGGACAATGGTCACAATCGAGGGACCTSGTTT
K K L G L R C H S K G T M V T I E G P X F

TAGCTCCCGGGCAGAAAGCTTCATGTTCCGCACCTGGGGGGCGGATGTTATCAACATGACCAC
S S R A E S F M F R T W G A D V I N M T T

AGTTCCAGAGGTGGTTCTTGCTAAGGAGGCTGGAATTTGTTACGCAAGTATCGCCATGGCGACA
V P E V V L A K E A G I C Y A S I A M A T

*GATTATGACTGCTGGAAGGAGCACGAGGAAGCAA*TG<u>ATCAAGTTCCAGATGATCCTCAGTGAG</u>
D Y D C W K E H E E A M I K F Q M I L S E

<u>GGATACCATCCTTTCMATATTCAAGAGTCACCCTTCTACAGAGGACTCCTAGACTTCCCATCA</u>
G Y H P F X I Q E S P F Y R G L L D F P S

<u>GTGGGRCATGGCAGAGGTGAAATCCTGCCCCTGTCTCCCTTAGACCTGGCTRGATACTGCTTCC</u>
V G H G R G E I L P L S P L D L A X Y C F Q

<u>AACAACCCATGCAGCCACCCTGCCCTGACAGCTAG*CAAGAGGCCMAGAMCCACAGAACAA</u>
Q P M Q P P C P D S *

<u>TCACCATCACACCTCTGTCNGCAGGAAGCAGTTACAGAAGACTGACCTTCATCCAGTTTCCCAAAGA</u>

<u>ATTGGGTCATGGATTTTTGCGGGGGAAAACGTTAGAGTAGGTAATTAGGCAGACATGAGTAAGGGA</u>

<u>GGAGAGACCCCCTCCAACTAGGAATGTCAGGTGAGCATCAGATGATCATCAGGTGGTTGTTAAAC</u>

<u>TCTCTCTCTAAAATAATAATAGGTTGCAACTGGCAGCAGGGAAAGACAATCTCCATCTCCCAAT</u>

<u>AGATAGAAAAGTCCTGAAGCTGGTGATCAGCAGCTTCCTAGTAAGATCTCAGGATTTGGGCAA</u>

<u>GCAGGCTCAAGCATGGGCACTAAGAGGCAAAATCGTGGAGTTTANCTGGTATACAGACTTCCT</u>

<u>CTACAAACACATGACTCATGCACATGTGGACAGACTGCC</u>

Fig. 6. Sequence of the total 1.2 Kb cDNA. Region homologous to *MTAP* is in italics. Region homologous to *ALA* is underlined. The base pair that begins the sequence identity to c110 is underlined. Stop signal for the open reading frame is indicated (*). Translation of the open reading frame is below. The region of identity to 807E4-2 is bold and underlined. The primer sequence from 807E4-2 used to amplify the cDNA is the last 20 base pairs of the sequence. Genbank accession number AF216650.

MTAP sequences or whether it is simply a splice variant of the *MTAP* gene (the 7 Kb transcript). In either case, the predicted protein of this transcript would replace the carboxy terminal 67 amino acids of *MTAP* with 63 new amino acids, and would alter the 3′ untranslated region. We do not know the transcriptional start site; therefore, it is possible that novel 5′ sequences could also alter the function of the protein product.

In our small sample of tumor cell lines, we detected the absence of one particular transcript (7 Kb) of the *MTAP*/1.2 Kb cDNA from tumor cell line mRNA more frequently than any of the other transcripts (Fig. 5). The presence or absence of the 8.5 Kb transcript could not be unequivocally determined in this study. It is possible that the 7 Kb transcript is specifically targeted for alteration because of a function unrelated to *MTAP*. However, primary tumor tissue must be used to determine the presence or absence of the full length 7 and 8.5 Kb transcripts before the implications of our data can be assessed.

One of *MTAP* functions is to catalyze a reaction in the purine nucleotide salvage pathway [30], and retention of this function would seem to give a selective advantage to a rapidly growing tumor cell. However, the *MTAP* gene is co-deleted with *CDKN2A* in approximately 80–90% of tumors [31], which is a higher frequency than the closely linked *CDKN2B* gene. There is precedence in the literature for genes with alternative splice products that have opposing functions. One such example is BCLx1 and BCLxs, where the former antagonizes apoptosis and the latter promotes apoptosis [32]. In addition, there is an example of another "housekeeping" enzyme (NM23/NDP kinase), that catalyze nuclotide phosphoral transfer and but also has distinct functions in cell differentiation, apoptosis and suppression of metastatic spread [33]. The higher frequency of alteration of the 7 Kb transcript relative to the other *MTAP* transcripts may indicate that the protein product derived from the 7Kb transcript has a unique function, and the elimination of which augments tumorigenesis.

We saw strong sequence homologies of the cDNA with cosmid clones from other chromosome bands such as 7q21~q22, 7p15~p21, 14q24 (15 Kb from the *TGF-B 3* gene) and Xq21 (13 Kb downstream from a putative CpG island). The strongest homology is seen with the 7q21 region. Interestingly, this region is the site of many chromosome abnormalities including deletions in myeloid leukemia [34]. The *ALA* gene maps near the Abelson locus on chromosome 9q, which is also a frequent site of chromosome translocation in leukemia [35]. Although the 3.5 Kb cDNA we selected did not map to our region, the 9 Kb transcript that it identified was absent in 66% of our tumor samples (data not shown). This was particularly striking because the transcript was present in every cell type except PBL. It is possible that genes with these homologous sequences are targeted for alteration in cancer cells, or that they possess a structural feature that results in genomic instability. In either case, the isolation and analysis of these sequences are difficult due to their apparent abundance in the genome. This problem emphasizes the critical importance of the ESS procedure in the isolation of a 9p21-specific cDNA. Successful cloning of this gene will add to our understanding of an important pathway in tumorigenesis.

The data presented here describes a gene located in genetically interesting locus with a complex tissue expression and targeted alterations in tumor cells. Our data suggests that the 1.2 Kb cDNA we have identified is a good candidate for further investigation in the search for an additional TSG on chromosome band 9p21.

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